Recombinant BCG Strains Expressing Alanine Dehydrogenase, Serine Dehydratase and/or Glutamine Synthetase as TB vaccines

Field of the Invention

This invention relates to tuberculosis (TB) vaccines.

Background of the Invention

TB is a deadly contagious disease caused by the infectious agent, *Mycobacterium tuberculosis*. It kills 2 million people each year. The World Health Organization (WHO) 2001 annual report estimated that there would be 8.4 million new TB cases in 1999, up from 8.0 million in 1997. If the present trend continues, it is estimated that between 2000 and 2020, nearly one billion people will be newly infected, 200 million people will become ill and 35 million will die from TB. The spread of HIV/AIDS and the emergence of multidrug-resistant TB contribute to the worsening impact of this disease. Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is currently the only available vaccine for the prevention of TB. In animal models of infection, BCG vaccination has been demonstrated to induce protective immunity against a *M. tuberculosis* challenge (Baldwins et al., 1998). In humans, BCG vaccination has demonstrated consistent protection against the childhood forms of TB, especially meningitis. However, BCG vaccination is controversial due to variations in its efficacy for protecting adults from pulmonary TB (Fine, 1989; Colditz et al., 1994; Sterne et al., 1998). Trials conducted in the 1940s and 1950s in developed countries such as the United Kingdom, Denmark and North America demonstrated the vaccine to be highly efficient (70-80%). However, in the single largest clinical trial, which took place in India in 1970s and involved more than 265,000 persons, BCG vaccination provided no detectable protection against pulmonary TB. Thus, there is an urgent need to generate an improved vaccine(s) to replace the BCG and to prevent TB.

Several explanations have been suggested for the variation in protective efficacy of BCG (Andersen, 2001). The most prominent hypothesis is that exposure to environmental mycobacteria sensitizes the host against mycobacteria in general, thereby providing

heterologus immunity that obscures the potential benefits of BCG vaccination (Fine, 1995; Fine and Vynnycky, 1998). Furthermore, a recent study showed that the multiplication of BCG was inhibited in animals sensitized with environmental mycobacteria, and consequently BCG vaccination elicited only a transient immune response and failed to provide protective immunity against TB (Brandt et al., 2002). This study also supports the long-standing observation that the induction of immunity to TB requires productive infection by BCG. BCG is a live vaccine; killed BCG does not provide protection. Like *M. tuberculosis*, BCG is capable of forming granulomas and abscesses in various tissues in the infected host (Hogan et al., 2001). The ability of *M. tuberculosis* and *M. bovis* BCG to survive and persist within granulomas, a hostile environment with restricted access to nutrients and reduced oxygen tension, appears to be dependent on the ability of the bacteria to adapt their metabolism to the available source of carbohydrate, nitrogen, and energy (Barclay and Wheeler, 1989). A recent study revealed that fatty acids serve as a source of carbohydrates and are required for persistence of *M. tuberculosis* in mice and activated macrophages (McKinney et al., 2000). Following vaccination in immunocompetent individuals, BCG may persist for certain periods before it is eliminated from the host (Dunn and North 1995; Lagranderie et al., 1996; Moisan et al., 2001).

The key to developing a new and effective TB vaccine is to provide long-term protection (Orme, 2001; Young, 2000). Existing BCG vaccines impart protection against the manifestations of TB in children, but their efficacy wanes over a period of 10 to 15 years, presumably because the protective immunity induced by BCG is gradually lost (Orme, 2001). New strategies to developing an improved vaccine have included the use of attenuated mycobacteria, subunit vaccines and DNA vaccines (Andersen, 2001). However, none of these have proved to be more potent than, or even as effective as BCG. Survival and growth of *M. bovis* BCG is necessary for eliciting protective immunity. It has been shown that early treatment of infected mice with isoniazid to inhibit bacillary growth prevents the development of acquired resistance. BCG strains that persist for extended periods within the host are required in order to obtain more effective vaccines. As such, there is a need for novel, recombinant strains of Bacille Calmette-Guérin.

Summary of the Invention

The invention provides vaccines that overcome the limited ability of BCG strains to use naturally occurring amino acids as the nitrogen source for growth. Furthermore, L-alanine, D-alanine, or L-serine inhibits the growth of BCG strains even when ammonium is present. Expressing a functional alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO: 2] in BCG strains relieves the growth inhibition of BCG by alanine. Similarly, expressing a functional L-serine dehydratase [SEQ ID NO:5; SEQ ID NO: 6] in BCG strains relieves the growth inhibition of BCG by L-serine. The mechanism for such inhibition occurs through blockage of glutamine synthetase. Overexpression of glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] in BCG relieves the growth inhibition of BCG by alanine and L-serine. Recombinant BCG strains that express (or overexpress) a functional alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO: 2], a L-serine dehydratase [SEQ ID NO:5; SEQ ID NO: 6], and/or glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] survive and persist longer within the host and consequently induce long-term protective immunity. Such persistent recombinant BCG strains provide more effective vaccines for the prevention of TB and other mycobacterial infections.

The present invention relates to recombinant *Mycobacterium bovis* BCG, which express DNA encoding an alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO: 2], a L-serine dehydratase [SEQ ID NO:5; SEQ ID NO: 6], and/or a glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14]. We found that, due to the lack of a functional alanine dehydrogenase [SEQ ID NO:3; SEQ ID NO: 4], BCG cannot utilize alanine (L-alanine or D-alanine) as the only nitrogen source for growth. We further found that alanine (L-alanine or D-alanine) inhibits the growth of all BCG vaccine strains. Said inhibition is relieved by expressing a functional alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO: 2] in BCG. Similarly, BCG cannot utilize L-serine as the only nitrogen source for growth and that growth of BCG is inhibited by L-serine. Expressing a L-serine dehydratase [SEQ ID NO:5; SEQ ID NO: 6] in BCG strains relieves the growth inhibition by L-serine.

Alanine (L-alanine or D-alanine) and L-serine inhibits BCG growth likely by blocking the activity of glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14]. Overexpression of

glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] in BCG relieves the growth inhibition of BCG by alanine and L-serine. Glutamine synthetase, in conjunction with glutamate synthase, provides glutamine and glutamate, which are essential for biosynthesis of all amino acids, proteins, purines and pyrmidines. Inhibition of glutamine synthetase stops cell growth. Supplying amino acids that can be converted to glutamate such as L-glutamine, L-glutamate, L-aspartate, and L-asparagine can relieve such inhibition. Indeed, our data show that the inhibition of BCG growth by alanine (L-alanine or D-alanine) or L-serine is relieved by supplementing growth medium with L-glutamine, L-glutamate, L-aspartate, or L-asparagine.

Since BCG is a live vaccine, recombinant BCG strains expressing or overexpressing a functional alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO: 2], a L-serine dehydratase [SEQ ID NO:5; SEQ ID NO: 6], and/or a glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] survive longer within the human host and subsequently induce long-term memory immunity. These recombinant BCG strains provide extremely useful vaccines.

The present invention relates to a live recombinant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid encoding at least one protein or polypeptide that exhibits alanine dehydrogenase activity [SEQ ID NO:1; SEQ ID NO:2], glutamine synthetase activity [SEQ ID NO:7 to SEQ ID NO:14], or L-serine dehydratase activity [SEQ ID NO:5; SEQ ID NO:6].

The invention also relates to a live recombiant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid encoding at least one protein or polypeptide selected from the group consisting of alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2], glutamine synthetase [SEQ ID NO:7 to SEQ ID NO:14] and L-serine dehydratase [SEQ ID NO:5; SEQ ID NO:6].

The invention further relates to a live recombinant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid comprises all or part of at least one nucleic acid molecule selected from the group consisting of [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:5], [SEQ ID NO:6], [SEQ ID NO:7], [SEQ ID NO:8],

[SEQ ID NO:9], [SEQ ID NO:10], [SEQ ID NO:11], [SEQ ID NO:12], [SEQ ID NO:13] and [SEQ ID NO:14].

In one embodiment, the live recombinant *Mycobacterium bovis*-BCG strain is selected from the group consisting of *Mycobacterium bovis*-BCG-Russia, *Mycobacterium bovis*-BCG-Moreau, *Mycobacterium bovis*-BCG-Japan, *Mycobacterium bovis*-BCG-Sweden, *Mycobacterium bovis*-BCG-Birkhaug, *Mycobacterium bovis*-BCG-Prague, *Mycobacterium bovis*-BCG-Glaxo, *Mycobacterium bovis*-BCG-Denmark, *Mycobacterium bovis*-BCG-Tice, *Mycobacterium bovis*-BCG-Frappier, *Mycobacterium bovis*-BCG-Connaught, *Mycobacterium bovis*-BCG-Phipps, and *Mycobacterium bovis*-BCG-Pasteur.

Another aspect of the invention is a pharmaceutical composition comprising a live recombinant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid encoding at least one protein or polypeptide that exhibits alanine dehydrogenase activity [SEQ ID NO:1; SEQ ID NO:2], glutamine synthetase activity [SEQ ID NO:7 to SEQ ID NO:14], or L-serine dehydratase activity [SEQ ID NO:5; SEQ ID NO:6].

The invention also relates to a live recombiant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid encoding at least one protein or polypeptide selected from the group consisting of alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2], glutamine synthetase [SEQ ID NO:7 to SEQ ID NO:14] and L-serine dehydratase [SEQ ID NO:5; SEQ ID NO:6].

In yet another aspect of the invention there is a pharmaceutical composition comprising a live recombinant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid comprises all or part of at least one nucleic acid molecule selected from the group consisting of [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:5], [SEQ ID NO:6], [SEQ ID NO:7], [SEQ ID NO:8], [SEQ ID NO:9], [SEQ ID NO:10], [SEQ ID NO:11], [SEQ ID NO:12], [SEQ ID NO:13] and [SEQ ID NO:14].

In a futher aspect of the invention there is a vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by mycobacteria comprising a live recombinant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid encoding at least one protein or polypeptide that exhibits alanine dehydrogenase activity [SEQ ID NO:1; SEQ ID NO:2], glutamine synthetase activity [SEQ ID NO:7 to SEQ ID NO:14], or L-serine dehydratase activity [SEQ ID NO:5; SEQ ID NO:6].

In another aspect of the invention there is a vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by mycobacteria comprising a live recombiant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid encoding at least one protein or polypeptide selected from the group consisting of alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2], glutamine synthetase [SEQ ID NO:7 to SEQ ID NO:14] and L-serine dehydratase [SEQ ID NO:5; SEQ ID NO:6].

In yet another aspect of the invention there is a vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by mycobacteria comprising a live recombinant *Mycobacterium bovis*-BCG strain comprising a nucleic acid capable of expression, the nucleic acid comprises all or part of at least one nucleic acid molecule selected from the group consisting of [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:5], [SEQ ID NO:6], [SEQ ID NO:7], [SEQ ID NO:8], [SEQ ID NO:9], [SEQ ID NO:10], [SEQ ID NO:11], [SEQ ID NO:12], [SEQ ID NO:13] and [SEQ ID NO:14]. In a preferred embodiment the vaccine or immunogenic composition is for the treatment or prophylaxis of a mammal against challenge by *Mycobacterium tuberculosis*. In another preferred embodiment the vaccine or immunogenic compositions of the current invention further comprise a pharmaceutically acceptable carrier. In yet another preferred embodiment the vaccine or immunogenic compositions further comprise adjuvants. In a another embodiment the vaccine or immunogenic compositions further comprises immunogenic materials from one or more other pathogens.

Another aspect of this invention relates to a method for treatment or prophylaxis of a mammal against challenge by *Mycobacterium tuberuclosis* or *Mycobacterium bovis* comprising administering to the mammmal a vaccine or immunogenic composition of the instant invention. In one embodiment the mammal is a cow. In another embodiment the mammal is a human. In yet another embodiment the vaccine or immunogenic composition is administered in the presence of an adjuvant.

A further aspect of the invention is a method for the treatment or prophylaxis of a mammal aginst cancer comprising administering to the mammal a vaccine or immunogenic composition of the current invention. In one embodiment the cancer is bladder cancer. In another embodiment the vaccine or immunogenic composition is administered in the presence of an adjuvant.

The invention also relates to a test kit comprising the live recombinant *Mycobacterium bovis*-BCG strain of the instant invention.

The invention further relates to a media composition for inhibiting the growth of *Mycobacaterium bovis*-BCG comprising alanine as the only nitrogen source for growth. In another embodiment serine is the only nitrogen source for growth. In another embodiment, the media compositions of the current invention further comprise a carbon source, iron, magnesium, and SO_4. In one embodiment the carbon source is selected from the group consisting of glycerol, dextrose, citrate, and glucose.

The current invention relates to a method for inhibiting the growth of *Mycobacterium bovis*-BCG comprising the steps of (a) obtaining a sample comprising *Mycobacterium* and (b) culturing the sample in a selective media. In one embodiment the selective media comprises alanine as the only nitrogen source. In yet another embodiment the selective media comprises serine as the only nitrogen source.

Another aspect of the invention relates to a method for culturing *Mycobacterium bovis*-BCG comprising the steps of (a) obtaining a sample comprising *Mycobacterium* and (b)

culturing the sample in differential media. In one embodiment the differential media comprises histidine.

Brief Description of the Drawings

Preferred embodiments of the invention will be described in relation to the drawings in which:

Fig. 1. Cloning of the *ald* gene. First, a 4.5 kb *Sca*I fragment of *M. tuberculosis* genomic DNA containing the *ald* gene [SEQ ID NO:1] was ligated to *Ecl*136II-linearized pUC19 to generate pUC-ALD. Then, mycobacterial plasmid pALD was created by ligating the 1.9 kb *Kpn*I fragment containing the *ald* gene [SEQ ID NO:1] to *Kpn*I-linearized pMD31.

Fig. 2. Cloning of the *sdaA* gene.

Cloning of *sda*A [SEQ ID NO:5] was accomplished in two steps. First, a 9.5 kb *Bam*HI fragment of *M. tuberculosis* genomic DNA was ligated to *Bam*HI-linearized pMD31 to generate pSDA1. Plasmid pSDAA was generated by cleavage of pSDA1 with *Pst*I, followed by self-ligation of the 10.9 kb *Pst*I fragment.

Fig. 3. Inhibition of BCG growth by L-alanine in GAS. BCG-Japan, BCG-Frappier, and BCG-Pasteur grown to stationary phase in 7H9/ADC/glycerol/Tween-80 liquid media, were each inoculated into duplicated 5 ml culture volumes of GAS, GAS without L-alanine, and GAS supplemented with 27 mM L-asparagine, to a cell density of 2×10^7 cells/ml. Cultures were incubated at 37°C with constant shaking for 16 days and then 2 ml aliquots of cell culture were centrifuged and cell pellet lyophilized to determine cell dry weight.

Fig. 4. Inhibition of BCG growth by increasing concentrations of L-alanine in Sauton containing NH_4Cl (5 g/liter). a) BCG-Japan, **b)** BCG-Frappier, and **c)** BCG-Pasteur, grown to stationary phase in 7H9/ADC/glycerol/Tween-80 liquid media. Cells were washed and resuspended in Sauton basal medium (no nitrogen source).

Resuspended cells of each strain were inoculated into duplicate 5 ml culture volumes of Sauton media supplemented with NH_4Cl and increasing concentrations of L-alanine. Cultures were incubated at 37°C with constant shaking for 30 days and cell dry weight was determined.

Fig. 5. Inhibition of BCG growth by D-alanine in GAS. BCG-Japan, BCG-Frappier, and BCG-Pasteur grown to stationary phase in 7H9/ADC/glycerol/Tween-80 liquid media, were each inoculated into 5ml culture volumes of GAS in which L-alanine was replaced by D-alanine, GAS without L-alanine and, GAS (containing D-alanine) supplemented with 27 mM L-asparagine, to a cell density of 2×10^7 cells/ml. Cultures were incubated at 37°C with constant shaking for 13 days and cell dry weight was determined.

Fig. 6. Growth of recombinant BCG strains expressing alanine dehydrogenase [SEQ ID NO:1] in GAS medium. The growth of BCG-Frappier/*ald*, BCG-Pasteur/*ald*, BCG-Frappier/pMD31, BCG-Pasteur/pMD31, BCG-Frappier, and BCG-Pasteur were compared. Cells of each strain, grown to stationary phase in 7H9/ADC/glycerol/Tween-80 liquid media, were washed and resuspended in Sauton basal medium (no nitrogen source). Resuspended cells were inoculated into duplicate 5 ml culture volumes of GAS without L-alanine, GAS containing L-alanine and GAS in which L-alanine was replaced by D-alanine. Cultures were incubated at 37°C with constant shaking for 15 days and cell dry weight was then determined.

Fig. 7. Inhibition of BCG growth by L-serine in GAS. BCG-Japan, BCG-Frappier, and BCG-Pasteur grown to stationary phase in 7H9/ADC/glycerol/Tween-80 liquid media, were each inoculated into duplicate 5 ml culture volumes of GAS in which L-alanine was replaced by L-serine, GAS without L-alanine, and GAS (containing L-serine) supplemented with 27 mM L-asparagine, to a cell density of 2×10^7 cells/ml. Cultures were incubated at 37°C with constant shaking for 15 days and cell dry weight was then determined.

Fig. 8. Growth of recombinant BCG strains expressing L-serine dehydratase [SEQ ID NO:5] in GAS medium containing L-serine. The growth of BCG-Japan/*sdaA*,

BCG-Frappier/*sdaA*, BCG-Pasteur/*sdaA*, BCG-Japan, BCG-Frappier, and BCG-Pasteur were compared. Cells of each strain, grown to stationary phase in 7H9/ADC/glycerol/Tween-80 liquid media, were washed and resuspended in Sauton basal medium (no nitrogen source). Resuspended cells were inoculated into duplicate 5 ml culture volumes of GAS without L-alanine, GAS in which L-alanine was replaced by L-serine, and GAS (containing L-serine) supplemented with 27 mM L-asparagine. Cultures were incubated at 37°C with constant shaking for 15 days and cell dry weight was then determined.

Fig. 9. Alignment of A) nucleotide and B) amino acid sequences of the *ald* genes of *Mycobacterium tuberculosis* (*M. tb*) [SEQ ID NO:1; SEQ ID NO:2] *and Mycobacterium bovis* (*M. bovis*) [SEQ ID NO:3; SEQ ID NO:4] . The point deletion causing the frameshift mutation in *M. bovis ald* [SEQ ID NO:3] is indicated with an arrow. Nucleotide codons and amino acids affected by this mutation are highlighted.

Detailed Description of the Invention

BCG vaccine strains have a limited ability to utilize amino acids as the nitrogen source for growth. Furthermore, we found that naturally occurring amino acids L-alanine and L-serine inhibit the growth of BCG strains. Expressing a functional L-alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2] in BCG relieves the growth inhibition by alanine. Expressing of a functional L-serine dehydratase [SEQ ID NO:5; SEQ ID NO:6] in BCG relives the growth inhibition by L-serine. As well, overproduction of glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] relieves the growth inhibition by alanine and serine. These novel findings are significant because recombinant BCG strains that express (or overexpress) a functional alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2], a L-serine dehydratase [SEQ ID NO:5; SEQ ID NO:6] , and/or glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] will survive better within the human host, induce long-term memory immunity and provide for more effective vaccines to prevent TB, particularly for protecting against pulmonary TB in adults.

It has long been known that administration of killed BCG strains results in a weak and transient immune response. Protective immunity requires survival and replication of BCG

in the vaccinated host. This notion is reinforced by a recent study of an animal model of infection, which showed that prior exposure to live environmental mycobacteria blocked the multiplication of BCG in infected mice. Consequently BCG elicited only a transient immune response which failed to provide protective immunity against TB (Brandt et al., 2002). Live BCG continuously secrete many different antigens that are likely important for the induction of protective immunity. The continuous production of numerous antigens by multiplying BCG gives live vaccines an advantage over subunit vaccines or DNA vaccines which transiently produce a few antigens. Thus the ability of BCG to multiply and persist within the host is an important determinant of BCG efficacy.

In order to grow and persist within the host, BCG must be able to utilize the available nutrients inside the host. It was demonstrated that isocitrate lyase, an essential enzyme for catabolism of fatty acids, is required for persistence of *M. tuberculosis* during the chronic phase of infection and that this requirement was dependent on an intact immune response of the host (McKinney et al., 2000). In another study, an *M. bovis* BCG strain lacking anaerobic nitrate reductase, an enzyme essential for nitrate respiration, failed to persist in lungs, liver and kidneys of immune-competent mice (Fritz et al., 2002). Our findings, that BCG strains utilize only a few types of amino acids as the nitrogen source for growth, and that the growth of all BCG strains are inhibited by naturally occurring L-alanine and L-serine, suggest that the ability of BCG to grow and persist within the host is restricted. The concentration of L-alanine that is available to BCG growing in human is estimated to be 0.33-0.42 mM (Barclay and Wheeler, 1989), which is sufficient to inhibit the growth of BCG-Pasteur or BCG-Frappier, and significantly reduce the growth of BCG-Japan (Fig. 4). The concentration of L-serine present in the extracellular fluids of the host is around 0.1 mM (Barclay and Wheeler, 1989), which may cause significant inhibition of BCG growth. Since multiplication of BCG is required to generate protective immunity, such inhibition by amino acids within the host may prevent the development of long-term protective immunity and hence the lack of protection against pulmonary TB in adults.

M. bovis BCG is also used in the treatment of bladder cancer. Numerous randomized controlled clinical trials indicate that intravesical administration of BCG can prevent or delay tumour recurrence (reviewed in Lamm, 2000; Lockyer and Gillatt, 2001). The

details of how BCG exerts this effect remain to be determined. However, the antitumour response requires an intact T-cell response, and involves increased expression of Th1-type cytokines, including TNFα and IL-6 (reviewed in Prescott et al, 2000). The most effective treatment regimes involve multiple applications of BCG, which suggests that prolonged exposure to the bacteria is required. Similarly, tumours that retain the ability to phagocytize BCG are most susceptible to this treatment (de Boer et al 1996), indicating that bacterial interactions with the tumour are important. As such, a BCG strain demonstrating increased persistence may provide enhanced antitumour activity.

We show that the absence of a functional alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2] is responsible for the failure of BCG strains to utilize alanine (L-alanine or D-alanine) as the only nitrogen source. A gene (Rv2708) coding for a L-alanine dehydrogenase (*ald*) [SEQ ID NO:1] was identified in the genome of *M. tuberculosis*. The activity of this enzyme from *M. tuberculosis* had been demonstrated biochemically *in vitro*. Ald converts L-alanine to pyruvate and ammonium, and is highly specific for L-alanine (Hutter and Singh, 1999). This enzyme was detected in the culture supernatent fraction of *M. tuberculosis* but not in *M. bovis* BCG-Japan nor BCG-Copenhagen, even though DNA Southern blot showed that the gene is present in both BCG strains (Anderson et al., 1992). Similarly, we do not detect alanine dehydrogenase activity in any of the 12 BCG strains listed in this report (data not shown). This lack of a functional alanine dehydrogenase in BCG strains is probably caused by a mutation within the *ald* gene [SEQ ID NO:3], and probably originated with the original *M. bovis* strain. A frame-shift mutation is found within the *ald* gene in the published genome sequence of *M. bovis* (Fig. 9) [SEQ ID NO:3]. As a result, the full length L-alanine dehydrogenase protein [SEQ ID NO:2; SEQ ID NO:4] cannot be made in BCG strains and subsequently BCG cannot catabolize alanine. Similarly, the failure of BCG to utilize L-serine as the only nitrogen source is likely to be caused by either mutations or altered expression of the *sdaA* gene [SEQ ID NO:5; SEQ ID NO:6], which encodes L-serine dehydratase. Expression of *sdaA* [SEQ ID NO:5; SEQ ID NO:6] of *M. tuberculosis* in BCG allows BCG strains to grow on L-serine as the only nitrogen source and relieves the inhibition of BCG growth by L-serine (Fig. 8). The inhibition of BCG growth by alanine and serine is

caused by inhibition of glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14]. Overexpression of a glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] in BCG relieves the growth inhibition by L-serine, L-alanine and D-alanine.

BCG-Frappier and BCG-Pasteur are more susceptible than BCG-Japan to inhibition by alanine, presumably due to difference in the expression level or activity of glutamine synthetase. BCG-Japan differs from BCG-Frappier or BCG-Pasteur genetically (Behr et al., 1999). Calmette and Guérin developed the BCG vaccine in 1921 after 13 years and 230 passages of an isolate of *M. bovis in vitro*. Starting from 1924, BCG lots were distributed to laboratories around the world. These laboratories continued the passage of the bacteria *in vitro* employing a variety of different recipes and protocols until 1961when lyophilized seeds were established. As a consequence of such practices, different BCG progeny strains were created, which differed biochemically and genetically (Oettinger et al., 1999; Behr et al., 1999). Our data show that the ability of BCG strains to utilize amino acids as nitrogen source vary; for example, BCG-Japan is able to grow on cationic amino acids including L-arginine and L-lysine while BCG-Pasteur and BCG-Frappier cannot. These differences may also contribute to the differences of BCG efficacy in various clinical trials.

In summary, we use recombinant BCG strains that express (or overexpress) a functional alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2], a L-serine dehydratase [SEQ ID NO:5; SEQ ID NO:6], and/or glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO: 14] as vaccines to prevent TB and other mycobacterial infections. These recombinant BCG vaccines will induce long-term protective immunity against TB.

Variations of Nucleic Acid Molecules

Modifications

Many modifications may be made to the nucleic acid molecule DNA sequences disclosed in this application and these will be apparent to one skilled in the art. The invention includes nucleotide modifications of the sequences disclosed in this application (or fragments thereof) that are capable of directing expression in bacterial or mammalian

cells. Modifications include substitution, insertion or deletion of nucleotides or altering the relative positions or order of nucleotides.

Nucleic acid molecules may encode conservative amino acid changes in alanine dehydrogenase, glutamine synthetase or L-serine dehydratase. The invention includes functionally equivalent nucleic acid molecules that encode conservative amino acid changes within alanine dehydrogenase, glutamine synthetase or L-serine dehydratase and produce silent amino acid changes in alanine dehydrogenase, glutamine synthetase or L-serine dehydratase. Methods for identifying empirically conserved amino acid substitution groups are well known in the art (see for example, Wu, Thomas D. "Discovering Emperically Conserved Amino Acid Substitution Groups in Databases of Protein Families" (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=8877523&dopt=Abstract).

Nucleic acid molecules may encode non-conservative amino acid substitutions, additions or deletions in alanine dehydrogenase, glutamine synthetase or L-serine dehydratase. The invention includes functionally equivalent nucleic acid molecules that make non-conservative amino acid changes within the amino acid sequences in [SEQ ID NO:2, 6, 8, 10, 12, or 14]. Functionally equivalent nucleic acid molecules include DNA and RNA that encode peptides, peptides and proteins having non-conservative amino acid substitutions (preferably substitution of a chemically similar amino acid), additions, or deletions but which also retain the same or similar alanine dehydrogenase, glutamine synthetase or L-serine dehydratase activity as the alanine dehydrogenase shown in [SEQ ID NO:2], glutamine synthetase shown in [SEQ ID NO:8, 10, 12, or 14] or L-serine dehydratase shown in [SEQ ID NO:6].

The DNA or RNA can encode fragments or variants of alanine dehydrogenase, glutamine synthetase or L-serine dehydratase.

Fragments are useful as immunogens and in immunogenic compositions.

The alanine dehydrogenase, glutamine synthetase or L-serine dehydratase like-activity of such fragments and variants is identified by assays as described below.

Sequence Identity

The nucleic acid molecules of the invention also include nucleic acid molecules (or a fragment thereof) having at least about: 60% identity, at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity or, most preferred, at least 99% or 99.5% identity to a nucleic acid molecule of the invention and which are capable of expression of nucleic acid molecules in bacterial or mammalian cells. Identity refers to the similarity of two nucleotide sequences that are aligned so that the highest order match is obtained. Identity is calculated according to methods known in the art. For example, if a nucleotide sequence (called "Sequence A") has 90% identity to a portion of [SEQ ID NO: 1], then Sequence A will be identical to the referenced portion of [SEQ ID NO: 1] except that Sequence A may include up to 10 point mutations (such as substitutions with other nucleotides) per each 100 nucleotides of the referenced portion of [SEQ ID NO: 1].

Sequence identity (each construct preferably without a coding nucleic acid molecule insert) is preferably set at least about: 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity or, most preferred, at least 99% or 99.5% identity to the sequences provided in SEQ ID NO:1 to SEQ ID NO:14 or its complementary sequence). Sequence identity will preferably be calculated with the GCG program from Bioinformatics (University of Wisconsin). Other programs are also available to calculate sequence identity, such as the Clustal W program (preferably using default parameters; Thompson, JD et al., Nucleic Acid Res. 22:4673-4680), BLAST P, BLAST X algorithms, Mycobacterium avium BLASTN at The Institute for Genomic Research (http:tigrblast.tigr.org/), Mycobacterium bovis, M. Bovis BCG (Pastuer), M. marinum, M. leprae, M. tuberculosis BLASTN at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/Microbes/), M. tuberculosis BLAST searches at Institute Pasterur (Tuberculist) (http://genolist.pasteur.fr/TubercuList/), M. leprae BLAST searches at Institute Pasteur (Leproma) (http://genolist.pasteur.fr/Leproma/), M. Paratuberculosis BLASTN at Microbial Genome Project, University of Minnesota (http://www.cbc.umn.edu/ResearchProjects/Ptb/ and

http://www.cbc.umn.edu/ResearchProjects/AGAC/Mptb/Mptbhome.html), various BLAST searches at the National Center for Biotechnology Information – USA (http://www.ncbi.nlm.nih.gov/BLAST/) and various BLAST searches at GenomeNet (Bioinformatics Center – Institute for Chemical Research) (http://blast.genome.ad.jp/).

Since the genetic code is degenerate, the nucleic acid sequence in [SEQ ID NO:1] is not the only sequence which may code for a polypeptide having dehydrogenase activity; the nucleic acid sequences in [SEQ ID NO:7, 9, 11, and 13] are not the only sequences which may code for a polypeptide having glutamine synthetase activity; and the nucleic acid sequence in [SEQ ID NO:5] is not the only sequence which may code for a polypeptide having L-serine dehydratase activity. This invention includes nucleic acid molecules that have the same essential genetic information as the nucleic acid molecules described in [SEQ ID NO:1, 5, 7, 9, 11 and 13]. Nucleic acid molecules (including RNA) having one or more nucleic acid changes compared to the sequences described in this application and which result in production of the polypeptides shown in [SEQ ID NO:2, 6, 8, 10, 12 and 14] are within the scope of the invention.
Other functional equivalent forms of alanine dehydrogenase-, glutamine synthetase-, and l-serine dehydratase-encoding nucleic acids can be isolated using conventional DNA-DNA or DNA-RNA hybridization techniques.

Hybridization

The invention includes DNA that has a sequence with sufficient identity to a nucleic acid molecule described in this application to hybridize under stringent hybridization conditions (hybridization techniques are well known in the art). The present invention also includes nucleic acid molecules that hybridize to one or more of the sequences in [SEQ ID NO:1] to [SEQ ID NO:14] or its complementary sequence. Such nucleic acid molecules preferably hybridize under high stringency conditions (see Sambrook et al. Molecular Cloning: A Laboratory Manual, Most Recent Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). High stringency washes have preferably have low salt (preferably about 0.2% SSC) and a temperature of about 50-65 °C.

Vaccines

One skilled in the art knows the preparation of live recombinant vaccines. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The live immunogenic ingredients are often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants that enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn -glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80™ emulsion.

The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing a *Mycobacterium tuberculosis* antigenic sequence resulting from administration of the live recombinant *Mycobacterium bovis*-BCG vaccines that are also comprised of the various adjuvants. The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions,

suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In addition, the live recombinant *Mycobacterium bovis*-BCG vaccine administered in conjunction with other immunoregulatory agents, for example, immune globulins.
A subject of the present invention is also a multivalent vaccine formula comprising, as a mixture or to be mixed, a live recombinant *Mycobacterium bovis*-BCG vaccine as defined above with another vaccine, and in particular another recombinant live recombinant *Mycobacterium bovis*-BCG vaccine as defined above, these vaccines comprising different inserted sequences.

Pharmaceutical compositions

The pharmaceutical compositions of this invention are used for the treatmentment or prophylaxis of a mammal against challenge by *Mycobacterium tuberculosis* or *Mycobacterium bovis*. The pharmaceutical compositions of this invention are also used to treat patients having degenerative diseases, disorders or abnormal physical states such as cancer.
The pharmaceutical compositions can be administerd to humans or animals by methods such as tablets, aerosol administration, intratracheal instillation and intravenous injection.

Media Compositions

The media compositions of this invention for inhibiting the growth of *Mycobacterium bovis*-BCG comprise alanine or serine as the only nitrogen source. When alanine is the only nitrogen source it is present in an amount of at least 0.03mM and when serine is the only nitrogen source it is present in an amount of at least 0.03mM.
The media compositions may further contain carbon in an amount of about 1.35g/L to about 1.65g/L, preferably in an amount of at least 1.5g/L; iron in an amount of about 0.045g/L to about 0.055g/L, preferably in an amount of at least 0.05g/L; magnesium in an amount of about 0.45g/L to about 0.55g/L, prefereably in an amount of at least 0.5g/L; and SO_4 in an amount of about 0.045g/L to about 0.055g/L, preferebly in an amount of at least 0.05g/L.

Kits

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials, including the live recombinant *Mycobacterium bovis*-BCG strains of the instant invention, in suitable containers, along with the remaining reagents and materials required for the conduct of the assay, as well as a suitable set of assay instructions. Any immunological test format is contemplated, such as ELISA, Western blot, sandwich assay etc., which are well known to those skilled in the art.

Materials and Methods

Bacterial strains and culture conditions. Twelve *M. bovis* BCG strains: BCG-Japan, BCG-Russia, BCG-Moreau, BCG-Sweden, BCG-Birkhaug BCG-Frappier, BCG-Pasteur, BCG-Glaxo, BCG-Phipps, BCG-Tice, BCG-Denmark, and BCG-Prague were used in this study and were obtained from Dr. Marcel Behr (McGill University). The identities of these strains were described in detail previously (Behr et al., 1999). Middlebrook 7H9 medium (Difco) contains (per liter) ammonium sulfate, 0.5 g; L-glutamate, 0.5 g; sodium citrate 0.1 g; pyridoxine, 1 mg; biotin, 0.5 mg; disodium phosphate 2.5g; monopotassium

phosphate, 1 g; ferric ammonium citrate 40 mg; magnesium sulfate 50 mg; calcium chloride 0.5 mg; zinc sulfate 1 mg; copper sulfate, 1 mg; and glycerol, 2 ml; with 5 g of albumin (fraction V; bovine), 2 g of dextrose, and 0.05% Tween 80 added after sterilization. Sauton medium contains (per liter) L-asparagine, 4 g; monopotassium sulfate, 0.5 g; magnesium sulfate 0.5 g; ferric ammonium citrate 50 mg; citric acid, 2 g; zinc sulfate, 1 mg; and glycerol, 60 ml; with 0.05% Tween 80 added after sterilization. Glycerol-alanine-salts (GAS) medium contains (per liter) 2 g of ammonium chloride, 1 g of L-alanine, 0.3 g of Bacto Casitone (Difco), 4 g of dibasic potassium phosphate, 2 g of citric acid, 50 mg of ferric ammonium citrate, 1.2 g of magnesium chloride hexahydrate, 0.6 g of potassium sulfate, 1.8 ml of 10 M sodium hydroxide, and 10 ml of glycerol. Tween 80 was added to 0.05% after sterilization. BCG cultures were grown at 37°C with constant shaking for 3-4 weeks.

Cloning of *ald*. Cloning of *ald* [SEQ ID NO:1] was accomplished in two steps (Fig. 1). First, a 4.5kb *Sca*I fragment of *M. tuberculosis* genomic DNA containing *ald* was ligated to *Ecl*136II-linearized pUC19 to generate pUC-ALD. Then mycobacterial plasmid pALD was created by ligating the 1.9 kb *Kpn*I fragment containing the *ald* gene [SEQ ID NO:1] to *Kpn*I- linearized pMD31 (Yu et al., 1998). The plasmid pALD was introduced by electroporation into *M. bovis* BCG, and recombinant *M. bovis* BCG selected on Middlebrook 7H9 agar (Difco) supplemented with 10% oleic/albumin/dextrose/catalase (OADC) enrichment and 25 μg/ml kanamycin.

Cloning of *sdaA*. Cloning of *sda*A [SEQ ID NO:5] was accomplished in two steps. First, a 9.5 kb *Bam*HI fragment of *M. tuberculosis* genomic DNA was ligated to *Bam*HI-linearized pMD31 to generate pSDA1. Plasmid pSDAA was generated by cleavage of pSDA1 with *Pst*I, followed by self-ligation of the 10.9 kb *Pst*I fragment. The plasmid pSDAA was introduced by electroporation into *M. bovis* BCG, and recombinant *M. bovis* BCG selected on Middlebrook 7H9 agar (Difco) supplemented with 10% oleic/albumin/dextrose/catalase (OADC) enrichment and 25 μg/ml kanamycin.

Example 1

Growth of BCG strains in Glycerol-Alanine-Salts (GAS) medium. During the course of our studies, we found that BCG-Japan strain was able to grow in GAS medium, albeit slower than in 7H9 medium. BCG-Frappier and BCG-Pasteur could not grow in GAS medium, even after prolonged incubation (2 months). The growth of other BCG strains in GAS medium was also examined. The results are summarized in Table I, and show that BCG-Japan, BCG-Russia, BCG-Moreau, BCG-Sweden and BCG-Birkhaug were able to grow in GAS medium while BCG-Frappier, BCG-Pasteur, BCG-Glaxo, BCG-Phipps, BCG-Tice, BCG-Denmark, and BCG-Prague could not. This is an interesting observation since all 12 BCG strains listed above were able to grow in 7H9 and Sauton broth medium (Table I). To find out why certain BCG strains were unable to grow in GAS medium, the chemical compositions of GAS, 7H9 and Sauton medium were compared. Supplementing $ZnSO_4$ (1 mg/liter), which is present in 7H9 and Sauton but not in GAS medium, or sodium pyruvate (0.5%), which is required for growth of large colonies of *M. bovis*, did not support the growth of BCG strains in GAS (data not shown). Next, nitrogen sources were compared. L-Asparagine (4 g/liter) is the only nitrogen source in Sauton medium while ammonium chloride (2 g/liter) and L-alanine (1 g/liter) are the main nitrogen sources in GAS. When L-asparagine (at 4 g per liter) was added to GAS medium, BCG-Frappier, BCG-Pasteur, BCG-Glaxo, BCG-Phipps, BCG-Tice, BCG-Denmark, and BCG-Prague were able to grow rapidly (Table I). Supplementing L-aspartate, L-glutamine, or L-glutamate but not other types of amino acids to GAS medium also supported the growth of these BCG strains (Table I). These results show that the failure of certain BCG strains to grow in GAS medium is caused by their inability to utilize the nitrogen source present.

Example 2

Amino acids as the nitrogen source for growth of BCG strains. The above result prompted us to examine the ability of BCG strains to utilize various types of amino acids as the only nitrogen source. Since GAS medium contains a small amount of Bacto Casitone (0.3 g/liter), which is a complex mixture of various amino acids and peptides,

we chose Sauton medium, which is a defined medium, for this purpose. The L-asparagine in the original formula for Sauton medium was replaced individually by each type of amino acids at the same concentration (27 mM), and pH was adjusted to 7.0. Ammonium chloride at 27 mM or 1 mM as the only nitrogen source was also tested. Table II summarizes the results for three representative BCG strains, BCG-Japan, BCG-Pasteur, and BCG-Frappier. Consistent with the result in Table I, all three BCG strains grew rapidly when L-asparagine, L-aspartate, L-glutamine, or L-glutamate was used as the only nitrogen source. BCG-Japan was able to grow on cationic amino acids (e.g., L-arginine, L-lysine) while BCG-Pasteur and BCG-Frappier could not. More interestingly, none of the BCG strains were able to utilize L-alanine, L-serine, L-leucine, L-isoleucine, L-methioine, or L-glycine as the only nitrogen source, while other *Mycobacterium* species, including pathogenic *M. tuberculosis* and *M. avium,* and nonpathogenic *M. smegmatis*, were able grow on these amino acids. These results demonstrate that BCG vaccine strains utilize limited types of amino acids as the nitrogen source for growth; some BCG strains such as BCG-Pasteur or BCG-Frappier can grow only on 4 types of amino acids (Table II). Such a limitation is likely to restrict the ability of BCG to grow and persist *in vivo* (within the host).

Example 3

L-Alanine, D-alanine, or L-serine inhibits the growth of BCG. One surprising finding from the above experiment was that all BCG strains are able to grow on ammonium chloride as the only nitrogen source at both low (1 mM) or high concentrations (27 mM) (Table II). This is contradictory to the result obtained in GAS medium, in which ammonium chloride at 37 mM does not support the growth of BCG-Pasteur and BCG-Frappier (Table I). Since GAS medium also contains L-alanine, and L-alanine is not utilized by BCG strains for growth (Table II), the only possible explanation is that L-alanine actually inhibits the growth of BCG strains. To prove this, a modified GAS medium, in which L-alanine was omitted, was made and the growth of BCG strains in this medium was examined. As predicted, BCG-Frappier and BCG-Pasteur, which are unable to grow in the original GAS medium containing L-alanine, grew rapidly in GAS without L-alanine (Fig. 3). BCG-Japan also grew more rapidly in this L-alanine free

medium than in the original GAS medium (Fig. 3). The same results were obtained for the other nine BCG strains listed in this report.

To further confirm this result, increasing concentrations of L-alanine were added to Sauton medium containing ammonium chloride (5 g/liter) and the growth of BCG-Japan, BCG-Frappier and BCG-Pasteur was determined (Fig. 4). Strikingly, even at a very low concentration (0.25 mM), L-alanine completely inhibited the growth of BCG-Frappier and BCG-Pasteur. Although the growth inhibition of BCG-Japan was somewhat less severe, L-alanine at 0.5 mM significantly reduced its growth and at 8-16 mM the growth was completely inhibited (Fig. 4). Taken together, these results clearly demonstrate that L-alanine inhibits the growth of BCG strains. We further found that D-alanine also inhibits the growth of BCG strains. The presence of D-alanine in GAS medium stopped the growth of BCG-Pasteur and BCG-Frappier, and significantly reduced the growth of BCG-Japan (Fig. 5). Similarly, the presence of L-serine in GAS medium significantly inhibited the growth of BCG-Japan, BCG-Frappier, and BCG-Pasteur (Fig. 7).

Example 4

Expressing L-alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2] in BCG relieves the inhibition of BCG growth by L-alanine and D-alanine. Alanine is an excellent source of nitrogen for many *Mycobacterium* species including *M. tuberculosis*, *M. avium*, and *M. smegmatis*. D-Alanine degradation begins with racemization to L-alanine, which is then broken down to ammonium and pyruvate by L-alanine dehydrogenase. Interestingly, a functional L-alanine dehydrogenase was detected in *M. tuberculosis* and *M. smegmatis* but not in BCG-Japan or BCG-Copenhagen (Andersen et al., 1992; Hutter and Dick, 1998). We did not detect L-alanine dehydrogenase activity in any of the BCG strains listed in this study (data not shown). The failure of BCG strains to utilize L- or D- alanine as the only nitrogen source for growth is due to the lack of a functional L-alanine dehydrogenase. To prove this, the *ald* gene [SEQ ID NO:1] coding for L-alanine dehydrogenase [SEQ ID NO:2] in the *M. tuberculosis* genome was cloned into a shuttle vector and transformed into BCG-Frappier and BCG-Pasteur. The resulting recombinant BCG strains were tested for their ability to grow in GAS medium containing

L-alanine or D-alanine. Both recombinant strains, BCG-Frappier/*ald* and BCG-Pasteur/*ald*, grew rapidly in GAS medium containing either L-alanine or D-alanine (Fig. 6), while strains containing the cloning vector alone did not grow. This result shows that expression of a functional L-alanine dehydrogenase [SEQ ID NO:1; SEQ ID NO:2] in BCG strains relieves the growth inhibition of BCG by L-alanine and D-alanine.

Example 5

Expressing L-serine dehydratase [SEQ ID NO:5; SEQ ID NO:6] in BCG relieves the inhibition of BCG growth by L-serine. L-Serine is used by *M. tuberculosis*, *M. avium* and *M. smegmatis*, but not *M. bovis* BCG, as the only nitrogen for growth. The failure of BCG to utilize L-serine as the only nitrogen source is likely to be caused by either mutations on or altered expression of the gene encoding L-serine dehydratase, *sdaA* [SEQ ID NO:5] , in BCG. Expression of *sdaA* [SEQ ID NO:5; SEQ ID NO:6] of *M. tuberculosis* in BCG allows BCG strains to grow on L-serine as the only nitrogen source and relieves the inhibition of BCG growth by L-serine (Fig. 8).

Example 6

Inhibition of BCG growth by L-alanine, D-alanine and L-serine are likely to occur by blocking the activity of glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO:14] . Glutamine synthetase plays a central role in nitrogen metabolism in bacteria (Reitzer, 1996). Working in tandem with glutamate synthase, glutamine synthetase catalyzes the synthesis of glutamine and glutamate, which together provide nitrogen for almost all amino acids, proteins, and nucleotides. In *Escherichia coli* and *Klebsiella aerogenes*, glutamine synthetase is under feedback inhibition – purified glutamine synthetase is inhibited by L-alanine, L-serine and glycine (Reitzer, 1996). Glutamine synthetase was identified as an extracellular protein in *M. tuberculosis* and *M. bovis* BCG (Harth et al., 1994). It is likely that undegraded L-alanine inhibits glutamine synthetase and subsequently prevents the growth of BCG. If this were correct, then L-serine, which was not catabolized by BCG for growth (Table I), would also inhibit the growth of BCG by the same mechanism. Supporting this hypothesis, addition of L-serine to GAS medium containing only ammonium chloride as the nitrogen source inhibits the growth of BCG-

Frappier, BCG-Pasteur or BCG-Japan (Fig. 7). Furthermore, if glutamine synthetase were the target of L-alanine and L-serine inhibition, then supplementing amino acids that can be converted to glutamate would also alleviate their effects, as demonstrated in *K. aerogenes* (Janes and Bender, 1998). Indeed, addition of L-glutamate and amino acids that could be catabolized to yield glutamate (L-glutamine, L-asparagine, and L-aspartate) allows the growth of BCG strains in the presence of alanine (Table I), but those that could not be catabolized to glutamate (e.g., L-lysine, L-methioine, L-leucine) fail to allow growth. BCG-Frappier and BCG-Pasteur are more sensitive than BCG-Japan to inhibition by alanine and serine, this is due to differences in the expression level or activity of glutamine synthetase [SEQ ID NO:7] to [SEQ ID NO:14], i.e., BCG-Japan produces more glutamine synthetase or with higher activity than BCG-Frappier or BCG-Pasteur.

The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made without departing from the spirit and scope thereof. For example, where the application refers to proteins, it is clear that peptides and polypeptides may often be used. Likewise, where a gene is described in the application, it is clear that nucleic acids or gene fragments may often be used.

All publications (including Genbank entries), patents and patent applications are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table I

Comparative growth of *M. tuberculosis*, *M. smegmatis* and *M. bovis* BCG substrains in 7H9, Sauton, and glycerol-alanine-salts (GAS) medium.

Mycobacterium[a]	7H9	Sauton	GAS	GAS + L-Asn[b]	GAS + L-Asp[b]	GAS + L-Glu[b]	GAS + L-Gln[b]
M. tuberculosis[c]	+	+	+	+	+	+	+
M. smegmatis	+	+	+	+	+	+	+
BCG-Russia	+	+	+	+	+	+	+
BCG-Moreau	+	+	+	+	+	+	+
BCG-Japan	+	+	+	+	+	+	+
BCG-Sweden	+	+	+	+	+	+	+
BCG-Birkhaug	+	+	+	+	+	+	+
BCG-Prague	+	+	-	+	+	+	+
BCG-Glaxo	+	+	-	+	+	+	+
BCG-Denmark	+	+	-	+	+	+	+

Mycobacterium[a]	7H9	Sauton	GAS	GAS + L-Asn[b]	GAS + L-Asp[b]	GAS + L-Glu[b]	GAS + L-Gln[b]
BCG-Tice	+	+	-	+	+	+	+
BCG-Frappier	+	+	-	+	+	+	+
BCG-Phipps	+	+	-	+	+	+	+
BCG-Pasteur	+	+	-	+	+	+	+

[a] Each 5 ml culture inoculated with 1×10^7 cells of *M. smegmatis* or *M. bovis* BCG substrains.

[b] L-Asn, L-Asp, L-Glu and L-Gln in GAS supplemented to a final concentration of 27 mM.

[c] Based on research literature.

Table II

Comparative growth of *M. bovis* BCG-Japan, BCG-Frappier, BCG-Pasteur, *M. tuberculosis*, *M. avium* and *M. smegmatis*

Media[a]	BCG-Japan[b]	BCG-Frappier[b]	BCG-Pasteur[b]	*M. tuberculosis*[c]	*M. avium*[c]	*M. smegmatis*[b]
Sauton basal	-	-	-	-	-	-
Group 1						
Sauton + L-Asn	+++	+++	+++	+++	+++	+++
Sauton + L-Asp	+++	+++	+++	+++	+++	+++
Sauton + L-Glu	+++	+++	+++	+++	+++	+++
Sauton + L-Gln	+++	+++	+++	+++	+++	+++
Sauton + L-Cys	+++	+++	+++	+++	+++	+++
Sauton + NH_4Cl	+++	+++	+++	+++	+++	+++
Group 2						
Sauton + L-Arg	++	-	-	+++	+++	+++
Sauton + L-His	++	-	-	+++	+++	+++

Media[a]	BCG-Japan[b]	BCG-Frappier[b]	BCG-Pasteur[b]	*M. tuberculosis*[c]	*M. avium*[c]	*M. smegmatis*[b]
Sauton + L-Lys	++	-	-	NA	+++	+++
Sauton + L-Pro	++	-	-	NA	-	+++
Sauton + GABA	++	-	-	NA	NA	+++
Sauton + L-Ornithine	++	-	-	NA	NA	+++
Group 3						
Sauton + L-Ala	-	-	-	+++	+++	+++
Sauton + L-Ser	-	-	-	+++	+++	+++
Sauton + L-Leu	-	-	-	+++	+++	+++
Sauton + L-Ile	-	-	-	+++	+++	+++
Sauton + L-Met	-	-	-	NA	+++	+++
Sauton + Glycine	-	-	-	+++	NA	+++
Group 4						
Sauton + L-Trp	-	-	-	-	-	-

Media[a]	BCG-Japan[b]	BCG-Frappier[b]	BCG-Pasteur[b]	*M. tuberculosis*[c]	*M. avium*[c]	*M. smegmatis*[b]
Sauton + L-Phe	-	-	-	+++	-	-
Sauton + L-Tyr	-	-	-	-	-	-
Sauton + L-Val	-	-	-	NA	-	-
Sauton + L-Thr	-	-	-	NA	-	-

[a] All amino acids, L-Ornithine and GABA supplemented to final concentration of 27mM. NH_4Cl was tested at 1mM, 27 mM and 96 mM.

[b] Each 5 ml culture inoculated with 1×10^7 cells of *M. smegmatis* or *M. bovis* BCG substrains.

[c] Based on research literature.

Reference

Andersen, A.B.F., P.F. Andersen, and L. Ljungqvist. 1992. Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis*. *Infect. Immun.* **60**:2317-23.

Andersen, P. 2001. TB vaccines: progress and problems. *Trends Immunol.* **22**:160-8.

Baldwin, S.L.F., C.F. D'Souza, A.D.F. Roberts, B.P.F. Kelly, A.A.F. Frank, M.A.F. Lui, J.B.F. Ulmer, K.F. Huygen, D.M.F. McMurray, and I.M. Orme. 1998. Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. *Infect. Immun.* **66**:2951-9.

Behr, M.A.F., M.A.F. Wilson, W.P.F. Gill, H.F. Salamon, G.K.F. Schoolnik, S.F. Rane, and P.M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* **284**:1520-3.

Brandt, L., C.J. Feino, O.A. Weinreich, B. Chilima, P. Hirsch, R. Appelberg, and P. Andersen. 2002. Failure of the *Mycobacterium bovis* BCG Vaccine: Some Species of Environmental Mycobacteria Block Multiplication of BCG and Induction of Protective Immunity to Tuberculosis. *Infect. Immun.* **70**:672-678.

Barclay, R. and P. R. Wheeler. 1989. Metabolism of mycobacterium in tissues, p. 37-106. *In* C. Ratledge, J. Stanford, and J. M. Grange (ed.), Clinical aspects of mycobacterial disease. Academic Press, London, United Kindom.

Brosch, R.F., S.V.F. Gordon, C.F. Buchrieser, A.S.F. Pym, T.F. Garnier, and S.T. Cole. 2000. Comparative genomics uncovers large tandem chromosomal duplications in *Mycobacterium bovis* BCG Pasteur. *Yeast* **17**:111-23.

Colditz, G.A.F., T.F.F. Brewer, C.S.F. Berkey, M.E.F. Wilson, E.F. Burdick, H.V.F. Fineberg, and F. Mosteller. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *JAMA* **271**:698-702.

de Boer, E.C., Bevers, R.F., Kurth, K.H., and Schamhart, D.H. 1996. Double fluorescent flow cytometric assessment of bacterial internalization and binding by epithelial cells. *Cytometry*. **25**:381-387.

Dunn, P.L.F. and R.J. North. 1995. Virulence ranking of some *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. *Infect. Immun*. **63**:3428-37.

Fine, P.E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* **346**:1339-45.

Fine, P.E. 1989. The BCG story: lessons from the past and implications for the future. *Rev. Infect. Dis.* **11**:S353-9.

Fine, P.E.F. and E. Vynnycky. 1998. The effect of heterologous immunity upon the apparent efficacy of (e.g. BCG) vaccines. *Vaccine* **16**:1923-8.

Fritz, C.F., S.F. Maass, A.F. Kreft, and F.C. Bange. 2002. Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. *Infect. Immun.* **70**:286-91.

Harth, G.F., P.C.F. Zamecnik, J.Y.F. Tang, D.F. Tabatadze, and M.A. Horwitz. 2000. Treatment of *Mycobacterium tuberculosis* with antisense oligonucleotides to glutamine synthetase mRNA inhibits glutamine synthetase activity, formation of the poly-L-glutamate/glutamine cell wall structure, and bacterial replication. *Proc. Natl. Acad. Sci.* USA **97**:418-23.

Harth, G.F., D.L.F. Clemens, and M.A. Horwitz. 1994. Glutamine synthetase of *Mycobacterium tuberculosis*: extracellular release and characterization of its enzymatic activity. *Proc. Natl. Acad. Sci.* USA **91**:9342-6.

Hogan, L.H.F., W.F. Markofski, A.F. Bock, B.F. Barger, J.D.F. Morrissey, and M. Sandor. 2001. *Mycobacterium bovis* BCG-induced granuloma formation depends on gamma interferon and CD40 ligand but does not require CD28. *Infect. Immun.* **69**:2596-603.

Hutter, B.F. and M. Singh. 1999. Properties of the 40 kDa antigen of *Mycobacterium tuberculosis*, a functional L-alanine dehydrogenase. *Biochem. J.* **343**:669-72.

Hutter, B.F. and T. Dick. 1998. Increased alanine dehydrogenase activity during dormancy in *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* **167**:7-11.

Janes, B.K.F. and R.A. Bender. 1998. Alanine catabolism in *Klebsiella aerogenes*: molecular characterization of the dadAB operon and its regulation by the nitrogen assimilation control protein. *J. Bacteriol.* **180**:563-70.

Lagranderie, M.R.F., A.M.F. Balazuc, E.F. Deriaud, C.D.F. Leclerc, and M. Gheorghiu. 1996. Comparison of immune responses of mice immunized with five different *Mycobacterium bovis* BCG vaccine strains. *Infect. Immun.* **64**:1-9.

Lamm, D.L. 2000. Efficacy and safety of bacille Calmette-Guerin immunotherapy in superficial bladder cancer. *Clin. Infect. Dis.* **31**(Suppl 3):S86-90.

Lockyer, C.R., and Gillatt, D.A. 2001. BCG immunotherapy for superficial bladder cancer. *J. R. Soc. Med.* **94**:119-23.

McKinney, J.D.F., z.B. Honer, E.J.F. Munoz-Elias, A.F. Miczak, B.F. Chen, W.T.F. Chan, D.F. Swenson, J.C.F. Sacchettini, W.R.J. Jacobs, and D.G. Russell. 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**:735-8.

Moisan, J.F., W.F. Wojciechowski, C.F. Guilbault, C.F. Lachance, S. Di Marco, E.F. Skamene, G.F. Matlashewski, and D. Radzioch. 2001.Clearance of infection with *Mycobacterium bovis* BCG in mice is enhanced by treatment with S28463 (R-848), and its efficiency depends on expression of wild-type Nramp1 (resistance allele). – *Antimicrob. Agents Chemother.* **45**:3059-64.

Oettinger, T.F., M.F. Jorgensen, A.F. Ladefoged, K.F. Haslov, and P. Andersen. 1999. Development of the *Mycobacterium bovis* BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. *Tuber. Lung Dis.* **79**:243-50.

Orme, I.M. 2001. The search for new vaccines against tuberculosis. *J. Leukoc. Biol.* **70**:1-10.

Prescott, S., Jackson, A.M., Hawkyard, S.J., Alexandroff, A.B., and James, K. 2000. Mechanisms of action of intravesical bacille Calmette-Guerin: local immune mechanisms. *Clin. Infect. Dis.* **31**(Suppl 3):S91-3.

Reitzer, L. J. 1996. Ammonium assimilation and the biosynthesis of glutamine, glutamate, aspartate, asparagine, L-alanine, and D-alanine, p. 380-390. *In* Neidhardt, F. C. (ed.), *Escherichia coli and Salmonella*, ASM Press, Washington, D.C.

Sterne, J.A., L.C. Rodrigues, and I.N. Guedes. 1998. Does the efficacy of BCG decline with time since vaccination? *International Journal of Tuberculosis & Lung Disease* **2**:200-207.

Young, D.B. 2000. Current tuberculosis vaccine development. *Clin. Infect. Dis.***30**:S254-6.

Yu, S.F., E.F. Fiss, and W.R.J. Jacobs. 1998. Analysis of the exochelin locus in *Mycobacterium smegmatis*: biosynthesis genes have homology with genes of the peptide synthetase family. *J. Bacteriol.* **180**:4676-85.